

Phylogenetic Placement of “Zoochlorellae” (Chlorophyta), Algal Symbiont of the Temperate Sea Anemone *Anthopleura elegantissima*

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Abstract. At northern latitudes the sea anemones *Anthopleura elegantissima* and its congener *A. xanthogrammica* contain unidentified green chlorophytes (zoochlorellae) in addition to dinophytes belonging to the genus *Symbiodinium*. This dual algal symbiosis, involving members of distinct algal phyla in one host, has been extensively studied from the perspective of the ecological and energetic consequences of hosting one symbiotic type over the other. However, the identity of the green algal symbiont has remained elusive. We determined the phylogenetic position of the marine zoochlorellae inhabiting *A. elegantissima* by comparing sequence data from two cellular compartments, the nuclear 18S ribosomal RNA gene region and the plastid-encoded *rbcL* gene. The results support the inclusion of these zoochlorellae in a clade of green algae that form symbioses with animal (*Anthopleura elegantissima*), fungal (the lichen genus *Nephroma*), and seed plant (*Ginkgo*) partners. This clade is distinct from the *Chlorella* symbionts of *Hydra*. The phylogenetic diversity of algal hosts observed in this clade indicates a predisposition for this group of algae to participate in symbioses. An integrative approach to the study of these algae, both within the host and in culture, should yield important clues about how algae become symbionts in other organisms.

Introduction

The sea anemone *Anthopleura elegantissima* (Brandt, 1835) is an important model organism for the study of

cnidarian-algal symbioses because it contains the most phylogenetically diverse algal symbionts known for marine cnidarians. At least two dinoflagellates (zooxanthellae, *Symbiodinium californium* and *S. muscatinei*; LaJeunesse and Trench, 2000) and green algae (zoochlorellae, *Chlorella*-like green cells in the phylum Chlorophyta; Muscatine, 1971; O'Brien and Wytttenbach, 1980) are photosynthetic symbionts of these clonal anemones. Zoochlorellae in *A. elegantissima* are restricted to northern latitudes (> 43 °N; Secord and Augustine, 2000) and co-exist with one of the two zooxanthellae (*S. muscatinei*; LaJeunesse and Trench, 2000). Experimental studies have shown that the composition of algal partners in *Anthopleura* spp. is influenced by light and water temperature (O'Brien and Wytttenbach, 1980; Saunders and Muller-Parker, 1997). Field observations of anemones in different microhabitats and transplant experiments (Bates, 2000; Secord and Muller-Parker, 2004) support the role of low light and low temperature in influencing the occurrence of zoochlorellae in anemone hosts.

The ecological consequences of harboring different symbionts have been explored through recent investigations comparing *S. muscatinei* and zoochlorellae with respect to photosynthesis and carbon translocation (Engebretson and Muller-Parker, 1999; Verde and McCloskey, 1996, 2001, 2002), occurrence of UV-absorbing mycosporine-like amino acids (Shick *et al.*, 2002), and predation on the anemone host (Seavy and Muller-Parker, 2002, and references therein). These studies have not demonstrated any significant negative effects of hosting zoochlorellae, although Verde and McCloskey's carbon budgets show that, compared to zooxanthellae, zoochlorellae translocate much less carbon to the host because of their presumed high growth rate (Verde and McCloskey, 1996).

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Although the dinoflagellate symbionts of cnidarian hosts have been extensively investigated (Baker, 2003; LaJeunesse *et al.*, 2003), the common green symbiont harbored by *A. elegantissima* has yet to be phylogenetically identified. Previous pigment analyses have verified the status of the green symbiont as a member of the Chlorophyta (Muscatine, 1971). Ultrastructural studies also indicated typical chlorophyte morphology. The cells have plastids with two surrounding membranes, abundant starch storage, and pyrenoids that are not penetrated by thylakoids. This coccoid green alga is very small—about 6–10 μm in diameter (O'Brien, 1978; Verde and McCloskey, 1996)—and the cells divide in two. It was presumed to be a species of marine *Chlorella*, but attempts to identify it have been hampered by the inability to culture isolates from the anemone host (pers. obs., G. Muller-Parker).

Our goal was to obtain sequence data that would identify the green symbiont found in *A. elegantissima*. As the most common algal symbionts in animals in freshwater associations (sponges, hydra) are members of the genus *Chlorella* (Reisser and Widowski, 1992), we were particularly interested in determining the phylogenetic relatedness of both freshwater and marine symbiotic green algae, universally called zoochlorellae. However, given the paraphyly of *Chlorella* (Huss and Sogin, 1990; Huss *et al.*, 1999), the identity of green anemone symbionts is uncertain.

Materials and Methods

Cell isolation

Zoochlorellate (green) specimens of *Anthopleura elegantissima* were collected on 25 July 2003, during low tide, from a rock wall (+0.65 m above mean lower low water [MLLW]) at Alexander Beach, Fidalgo Island, Washington (48° 29' 37" N, 122° 40' 52" W). Anemones were collected randomly from separate locations on the rock wall to avoid possible selection of clonemates. Specimens were transported to Western Washington University's Shannon Point Marine Center and maintained in a flow-through natural seawater table for a month. The anemones were fed once a week with freshly hatched brine shrimp nauplii. They were last fed 5 days before zoochlorellae were isolated from five small green anemones selected randomly from the stock maintained in the seawater table. The anemones were cleaned of any adhering debris by wiping their external surfaces with tissues and rinsing them thoroughly in filtered (>0.5 μm) seawater. The five anemones were chopped into pieces with a razor blade, and the pieces were combined into one sample. The sample was homogenized in cold filtered seawater by using a motorized pestle and 60-ml glass tissue homogenizer. The anemone homogenate was distributed into four 15-ml centrifuge tubes and centrifuged at 1500 g for 5 min to separate the algae (pellet) from the animal (supernatant) parts of the anemone. The algal pellets were

resuspended in filtered seawater and cleaned of remaining animal tissue by recentrifugation in additional washes of filtered seawater. After three rinses, the four algal pellets were combined into one 50-ml sample that was passed through a 30- μm Nitex mesh screen to remove animal clumps. The screened sample was centrifuged to concentrate the algae, and subsamples ($\sim 4.3 \times 10^6$ zoochlorellae, determined by hemacytometer counts) were placed into 1.5-ml microfuge tubes. The tubes were centrifuged to pellet the zoochlorellae, and the samples were lyophilized overnight using a VirTis Freezemobile 5 SL.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from the lyophilized algal cells by using a modified CTAB (cetyltrimethylammonium bromide) extraction that included grinding the algal cells in the presence of sterile sand, following Shoup and Lewis (2003). The *rbcL* region was amplified in two separate reactions with primers M34 and M740r and M636 and M1390r (Lewis *et al.*, 1997), and these fragments were sequenced directly using amplification and internal primers. Double-stranded polymerase chain reaction (PCR) products were sequenced in 10- μl volumes with the PRISM system (Applied Biosystems, Inc.) using the manufacturer's directions. Chromatograms from individual sequencing runs were trimmed, then assembled into consensus sequences in Sequencher (GeneCodes Corp., Inc.). Most (96%) of the reported base calls were verified with sequencing reactions in both the forward and reverse orientations; the remaining nucleotides were verified from three independent sequencing reactions in the same orientation. The resulting *rbcL* fragment was 1252 nucleotides in length.

The 18S region was PCR amplified with primers 284F and 1081R (Phillips and Fawley, 2000), resulting in an 802-nucleotide fragment. Because of the presence of a mixture of animal and algal DNA in the extraction, purified PCR fragments were cloned using the TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions. Ten colonies containing inserts were harvested. The presence of inserts of the predicted size was verified using PCR amplification with the M13 forward and reverse primers. The resulting PCR products were sequenced using 18S primers, yielding two clones containing algal PCR products.

The 18S and *rbcL* consensus sequences were subjected to BLAST searches (Altschul *et al.*, 1990) to screen for contaminant sequences prior to phylogenetic analyses. The 18S and *rbcL* sequences from the *A. elegantissima* green symbiont were deposited in Genbank under the numbers AY577786 and AY577787.

Phylogenetic analyses

To identify the closest matching sequences for inclusion in the phylogenetic analyses, the 18S and *rbcL* sequences

from the *A. elegantissima* green symbiont were compared to published sequences in NCBI using BLAST searches (Altschul *et al.*, 1990). These trebouxiophycean sequences and additional sequences from members of the class Trebouxiophyceae were included in the two alignments to better determine the phylogenetic placement of the symbiont. Available 18S sequences from other symbiotic green algae were also included. One of these included a partial (592-nucleotide) sequence of *Coccomyxa glaronensis*. Three species from the Chlorophyceae were used as a sister group to the Trebouxiophyceae. Foraminifera are known to contain green algal symbionts in the genus *Chlamydomonas* (Chlorophyceae); however, representative sequences from these taxa were not included in our analysis because they were characterized with ITS rDNA sequence data (Pawlowski *et al.*, 2001). Unlike the 18S alignment, the *rbcL* alignment contained many fewer sequences because of limited availability of green algal *rbcL* data in the literature, but it included sequences from the Ulvophyceae, Chlorophyceae, and Trebouxiophyceae. Genbank accession numbers from additional algal taxa are indicated by the species names in Figures 1 and 2. The final 18S alignment consisted of a total of 1774 characters, 112 of which were excluded from the analysis because they could not be aligned with certainty. Of the remaining 1662 characters, 191 were parsimony-informative. The final *rbcL* alignment contained 14 taxa and 1387 nucleotides, which included 429 parsimony-informative sites. The alignments will be available from TreeBASE (www.treebase.org).

Prior to phylogenetic analysis, each data set was analyzed using MODELTEST (Posada and Crandall, 1998). The favored substitution model for the 18S data was determined to be TIM + I + Γ . Parameter values were set as follows: relative base frequencies = ($\pi_A = 0.2567$, $\pi_C = 0.2147$, $\pi_G = 0.2783$, $\pi_T = 0.2503$), relative rate matrix = ($r_{AC} = 1.0000$, $r_{AG} = 2.6862$, $r_{AT} = 1.2968$, $r_{CG} = 1.2968$, $r_{CT} = 6.1421$, $r_{GT} = 1.0$), gamma shape = 0.6528, and proportion of invariant sites = 0.6168. The GTR + I + Γ model was determined to be best for the *rbcL* data set. Maximum likelihood parameter values were relative base frequencies = ($\pi_A = 0.2608$, $\pi_C = 0.1644$, $\pi_G = 0.2374$, $\pi_T = 0.3374$), relative rate matrix = ($r_{AC} = 0.7657$, $r_{AG} = 2.3752$, $r_{AT} = 6.2564$, $r_{CG} = 1.1691$, $r_{CT} = 8.7699$, $r_{GT} = 1.0$), gamma shape = 2.8267, and proportion of invariant sites = 0.5136.

All other portions of the phylogenetic analyses were performed using PAUP* 4.b.10 (Swofford, 2002) for UNIX. Maximum likelihood (ML) analysis used heuristic searches with 10 random additions of taxa, each followed by TBR branch swapping. Bootstrap analysis (Felsenstein, 1985) included 100 replicates, with a single random addition of taxa for each replicate, under the same model as was used for the heuristic searches. The maximum parsimony (MP) analysis included heuristic searches with 1000 random

additions of taxa, followed by TBR branch swapping. Characters were given equal weight and were unordered. Bootstrap analysis included 10,000 replicates, with a single random addition of taxa per replicate.

Results

As discussed by Huss *et al.* (1999), many evolutionarily distinct lineages of small asexual green algae are attributed to the genus *Chlorella*. While the green symbiont from *Anthopleura elegantissima* is phenotypically similar to *Chlorella* (Muscatine, 1971; O'Brien, 1978; Verde and McCloskey, 1996), it is not within the clade of *Chlorella sensu stricto* (Huss *et al.*, 1999). Using 18S data, the single ML tree (lnL score = -6641.10989) obtained (Fig. 1) indicated that the anemone alga is a member of the Trebouxiophyceae, and is in a lineage that contains other symbionts such as the isolate of *Coccomyxa glaronensis* obtained from the lichen *Nephroma* (Peltigerales) (Lohtander *et al.*, 2003), and the small green coccoids that are endophytic in the gametophytic tissues of *Ginkgo biloba* (Tremouillaux-Guiller *et al.*, 2002). The anemone alga is also distinct from species of *Trebouxia*, the most common genus of green photobionts in lichens (Friedl and Büdel, 1996), and another lichen symbiont in the genus *Myrmecia*. The MP analysis of 18S data resulted in 16 optimal trees (tree length = 770, CI = 0.5714). The strict consensus of these trees (not shown) differed from the ML tree in collapsing the weakly supported basal nodes in the ML tree (those indicated by circles) into a basal polytomy. However, the position of the *A. elegantissima* symbiont did not change; it was placed as before in a clade with *Coccomyxa* and *Paradoxia*, distinct from *Chlorella* and the *Hydra* symbionts.

Because the sequences of *Coccomyxa glaronensis* and those from the symbiont of *A. elegantissima* were partial 18S sequences, we also performed an MP analysis on the sites representing complete overlap of all sequences in the data set. This region corresponded to sites 264–618 in the full 18S alignment, for a total of 355 characters and 39 parsimony-informative sites. Heuristic searches, performed as for the full data set, produced 242 optimal trees (tree length = 102, CI = 0.7600). A strict consensus of these trees (not shown) was less resolved overall than that of the full data set. However, the zoochlorellae sequence grouped with sequences from *Paradoxia*, *Coccomyxa glaronensis*, and the *Ginkgo* endophytes, with a bootstrap value of 89.

Analyses of the *rbcL* data also place the green symbiont within the class Trebouxiophyceae, but separated from *Chlorella sensu stricto*. The single optimal ML tree (Fig. 2) had a lnL score of -7842.45399. These data were also analyzed using MP (not shown), which resulted in a single best tree (tree length = 1454, CI = 0.4986). This tree was identical to the ML tree except for the placement of the anemone symbiont near the base of the trebouxiophycean

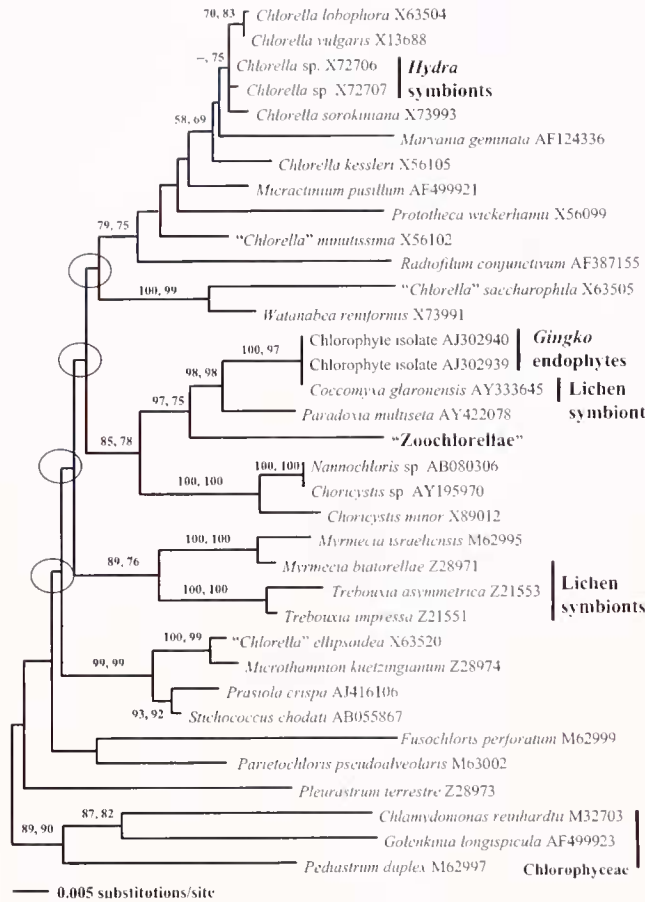


Figure 1. Phylogenetic tree obtained from a maximum likelihood analysis of 18S ribosomal RNA gene sequence from the green symbiont ("Zoochlorellae") of *Anthopleura elegantissima* and 34 additional green algae. With the exception of three sequences from members of the Chlorophyceae (indicated at base of tree by vertical bar), all of the sequences shown are from members of the Trebouxiophyceae. The positions of *Hydra* and lichen symbionts, and two *Ginkgo* endophytes, are also indicated. These sequences are from taxa that were isolated directly from their animal, plant, or fungal hosts. The nodes indicated by circles are those that collapse in the maximum parsimony analysis, leading to a basal polytomy. Numbers associated with nodes on the tree indicate maximum likelihood and maximum parsimony bootstrap proportions (out of 100 and 1000 replicates, respectively). Scale bar indicates the expected number of substitutions per site.

taxa, between *Prasiola* and a clade including the remainder of the Trebouxiophyceae and Chlorophyceae (indicated on Fig. 2). It is worthwhile emphasizing that there are currently a very limited number of published *rbcL* sequences of taxa in the Trebouxiophyceae; therefore, the placement of the "zoochlorellae" *rbcL* sequence is not close to any other taxa.

Discussion

Phylogenetic analysis of the nuclear-encoded 18S rDNA and plastid-encoded *rbcL* gene sequence data indicates that the green algal symbiont ("zoochlorellae") of *Anthopleura*

elegantissima obtained from our sampling is a member of the Trebouxiophyceae (Chlorophyta), a diverse lineage of mainly coccoid green algae that has free-living and symbiotic members. The "zoochlorellae" 18S sequence forms a well-supported clade with those obtained from the lichen symbiont *Coccomyxa glaronensis*, the small green endophytes of *Ginkgo biloba* (Tremouillaux-Guiller *et al.*, 2002), and with the sequence from *Paradoxia*, a free-living alga (Fig. 1). This clade is also related to very small free-living taxa such as *Nannochloris*. The anemone alga is distinct from species of *Trebouxia*, the most common genus of green photobionts in lichens (Friedl and Büdel, 1996), and another lichen symbiont in the genus *Myrmecia*. Although the sequences of *Paradoxia* and *Coccomyxa* are the closest to that of "zoochlorellae" on the 18S phylogenetic tree, these sequences are quite distinct. The corrected distances between the 18S sequences of "zoochlorellae" and *Coccomyxa* and *Paradoxia* are 0.01907 and 0.03933, respectively. These values are closer to the range observed within orders, such as between *Myrmecia* and *Trebouxia* in the Trebouxiiales, than for congeners of *Trebouxia* or *Chlorella* (data not shown).

The phylogenetic placement of zoochlorellae from *A.*

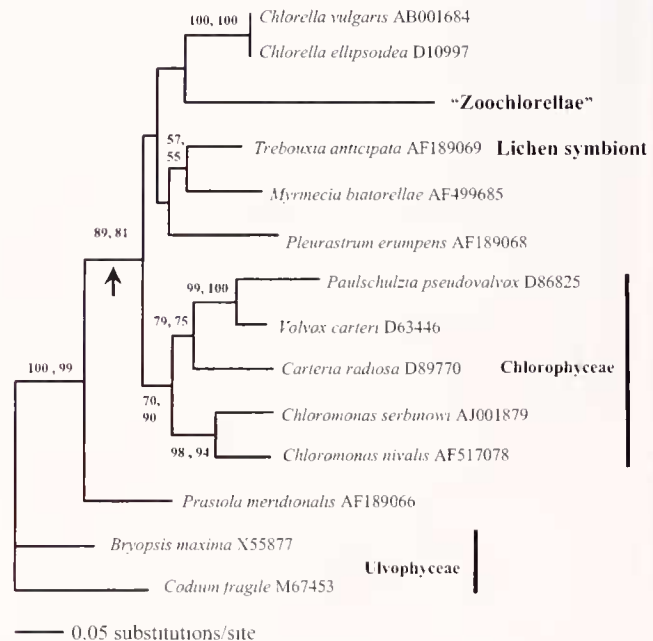


Figure 2. Phylogenetic tree obtained from a maximum likelihood analysis of *rbcL* data from the *Anthopleura elegantissima* green algal symbiont ("Zoochlorellae"), and published sequences from other members of the Trebouxiophyceae. Representative sequences of chlorophyceae and ulvophyceae algae were also included, as indicated by the vertical bars. One sequence of a green alga isolated from a lichen host is noted. The node indicated by an arrow shows the placement of the anemone symbiont in the maximum parsimony analysis. Numbers associated with nodes on the tree indicate maximum likelihood and maximum parsimony bootstrap proportions (out of 100 and 1000 replicates, respectively). Scale bar indicates the expected number of substitutions per site.

elegantissima in the same clade as a parasitic species of *Coccomyxa* (Fig. 1) is of particular ecological interest. Are zoochlorellae from *A. elegantissima* related to unicellular green algae that parasitize other marine animals? Algae are known to infect the epidermis of sea stars (Mortensen and Rosenvinge, 1910, 1933; pers. comm., R. Norris; all three cited in Stevenson, 1972). More recently, green algae from bivalve molluscs (mussels and scallops) have been identified as *Coccomyxa parasitica* (Stevenson and South, 1974; Gray *et al.*, 1999). However, the identification of this alga and of those that parasitize sea stars needs to be verified with molecular data, given the polyphyly of many phenotypically similar coccoid green algae (*e.g.*, Huss and Sogin, 1990; Lewis *et al.*, 1992; Potter *et al.*, 1997). In the echinoderms and molluscs, algal infection leads to tissue necrosis, loss of reproductive output, and eventual death. In all hosts, zoochlorellae maintain their photosynthetic pigments and, presumably, the ability to photosynthesize. In scallops, the algae are concentrated near shell margins where exposure to light is most likely (Stevenson, 1972). Muscatine (1971) raised the possibility that zoochlorellae are attempting to parasitize anemones and are tolerated because these hosts are capable of regulating algal populations by expelling excess algae. Perhaps the zoochlorellae in *Anthopleura* spp. are opportunistic symbionts, preempting space under conditions that promote their growth in anemones that form symbiotic associations with zooxanthellae. If so, this symbiosis may be fairly recent compared to that with zooxanthellae. Zoochlorellae persist in anemones under conditions that allow them to grow more rapidly than zooxanthellae. However, we do not know how these algae interact with each other and affect each other's growth rates under different environmental conditions. It will be difficult to advance our understanding of these interactions until we successfully culture both symbionts.

In 1881, Brandt introduced the term zoochlorella to describe the algae in freshwater hosts, including hydras, sponges and protists (Sapp, 1994). Since then, the term has been extended to include all green unicellular algae that inhabit animals. This term has no taxonomic value, as clearly the *Chlorella* symbionts of *Hydra* are phylogenetically distinct from the anemone symbiont (Fig. 1), and the genus *Chlorella* is now understood to be polyphyletic (Huss and Sogin, 1990; Huss *et al.*, 1999). Phylogenetic analyses using sequence data have indicated that green algae classified as *Chlorella* based on light microscopic features can be separated into many lineages within both the Chlorophyceae and Trebouxiophyceae. In fact, there are at least eight independent lineages that contain taxa previously classified as *Chlorella* (Huss *et al.*, 1999). *Chlorella sensu stricto* includes *C. vulgaris*, *C. lobophora*, *C. sorokiniana*, and *C. kessleri* (Trebouxiophyceae) and can be distinguished from other *Chlorella*-like taxa on the basis of its glucosamine-

containing cell walls, absence of carotenoids, and pyrenoids with thylakoid penetrations (Huss *et al.*, 1999).

We do not know if multiple strains or species of morphologically similar but genetically different zoochlorellae are distributed along a latitudinal gradient within their host's range, as are the two species of *Symbiodinium*. Our cloning showed only one kind of green alga from the five pooled anemones. Although we attempted to sample individuals randomly at the field site, we do not know if the five anemone hosts were genetically related (members of the same clone) and if host genetics influences the type of algal symbiont. Future studies should compare the genetic composition of populations of zoochlorellae obtained from *A. elegantissima* and from *A. xanthogrammica* at other locations, especially at the southern and northern limits of distribution of the algae. Such comparisons would be useful to determine whether the biogeographic trends observed among dinoflyte symbionts of cnidarians (Baker, 2003; LaJeunesse *et al.*, 2003) apply to symbiotic algae in other phyla. If, as we predict, different genotypes of zoochlorellae are distributed within the broad latitudinal range of their hosts, the nature of the symbiotic interactions between zoochlorellae, zooxanthellae, and the animal host, and the abilities of each partner to adapt to environmental change may differ according to geographic location.

The phylogenetic diversity of algal hosts observed in the trebouxiophycean clade containing the anemone symbiont, as illustrated in Figure 1, may indicate an inherited ability of this group of algae to participate in symbioses, making them good targets for the study of the initial establishment of a symbiotic association, host specificity, and nutritional interactions between the host and symbiont. For example, these algae may be predisposed to heterotrophy and low light conditions and able to survive long periods in the dark, and their free-living forms may differ in their growth and morphology. An integrative approach to the study of these algae, both within the host and in culture, should yield insight into the process of how algae become symbionts in other organisms.

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